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Activation of ERK1/2 by protein kinase C-α in response to hydrogen peroxide-induced cell death in human gingival fibroblasts

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ABSTRACT

Hydrogen peroxide (H_2O_2) increases protein tyrosine phosphorylation of numerous proteins in human gingival fibroblasts (HGFs). Two main proteins, with an apparent molecular weight of 44 and 42 kDa, were phosphorylated after hydrogen peroxide stimulation of the human gingival fibroblasts. Further analysis identified these two proteins as ERK1/2. Maximum phosphorylation was detected at 10 min post-H_2O_2 treatment. Pretreatment with an MEK inhibitor, PD98059, inhibited H_2O_2-stimulated ERK1/2 phosphorylation in a dose-dependent manner. Treatment with H_2O_2 also induced phosphorylation of protein kinase C-α (PKCα). Staurosporine, a PKC inhibitor, blocked ERK1/2 phosphorylation induced by H_2O_2. In addition, H_2O_2-induced cell death was prevented by PD98059, SB203580, and calphostin C, which are MEK, p38 and PKC inhibitors, respectively. These results suggest that H_2O_2 leads to the phosphorylation and activation of ERK1/2 in a PKC-dependent manner. These findings demonstrate that the MAPK signaling pathway plays an active role in mediating the H_2O_2-induced decrease in HGF cell viability and ATP depletion.

1. Introduction

In dentistry, hydrogen peroxide (H_2O_2) has been used primarily to enhance gingival healing after periodontal surgery, to reduce bacterial populations in dental plaque, and in tooth bleaching (Golub, 1989; Haywood, 1992; Hanks et al., 1993; Tipton et al., 1994). In H_2O_2-stimulated human gingival fibroblasts (HGFs), tyrosine phosphorylation is an early event that appears to play a key role in signal transduction. Reactive oxygen metabolites are increasingly being recognized for their ability to activate signal transduction pathways (Guyton et al., 1996; Schieven et al., 1994; Hardwick and Sefton, 1997; Pombo et al., 1997). Fibroblasts are the major cellular constituents of gingival connective tissue. They produce proteoglycans, hyaluronate, glycoproteins and collagen, as well as inflammatory cytokines, which play an important role in the pathogenesis of periodontitis (Bartold, 1987; Page, 1991).

The inflammatory response is frequently characterized by oxidant stress. This stress stimulates a variety of signal transduction pathways, including activation pathways of phospholipases (Min et al., 1998; Bai et al., 2002), phosphatases (Hecht and Zick, 1992; Sullivan et al., 1994) and nuclear factor-κB (NF-κB) transcription factor (Howe et al., 2002). When exposed to oxidants during the acute inflammatory response to bacterial infections, pleural mesothelial cells activate NF-κB and the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Milligan et al., 1996). Research done by Crossthwaite et al. (2002) has shown that the molecular mechanisms underlying oxidative stress induced neuronal damage and apoptotic mode of death is associated with members of the mitogen-activated protein kinase (MAPK) family, specifically ERK1/2.

Oxidants can trigger the activation of multiple signaling pathways including MAPKs (Morigasaki et al., 2008; Cieslak and Lazou, 2007). These kinases have been classified into three major subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase. The ERK pathway is mainly induced in response to mitogens and growth factors and plays a major role in regulating cell growth (Meyer et al., 2001; Li et al., 2000; Vepa et al., 1999), survival (Chen et al., 2008), and differentiation (Shimo et al., 2007). Several studies have reported that ERK activation prevents cell injury induced by a variety of cellular stresses, including oxidant stress (Hung et al., 2003), hypoxia, growth factor withdrawal, and chemotherapeutic agents (Ozaki et al., 2006). However, another study has reported that ERK activation contributes to H_2O_2-induced apoptosis (Ma et al., 2008). Some studies revealed that oxidative stress is implicated in pathological events leading to cell death. The enzyme poly (ADP-ribose) polymerase-1 belongs to a group of enzymes which catalyze poly(ADP-ribosyl)ation of

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damaged DNA to promote the repair process and because it is an energetically expensive event the cell depletes cellular nicotine adenine dinucleotide and ATP and consequently necrotica cell death (Huang et al., 2009).

The aim of this investigation was to determine whether H_2O_2 treatment promotes a decrease in cell viability and whether MAPKs are required. Here we report that treatment of HGFs with H_2O_2 results in ERK1/2 phosphorylation via a protein kinase C-α (PKCα)-dependent mechanism. These data demonstrate that the ERK and p38 pathways play an important role in H_2O_2-induced cell death and ATP depletion.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagles' medium (DMEM), foetal bovine serum (FBS), herbimycin A, and Hank's solution supplemented with penicillin/streptomycin/fungizone were obtained from invitrogen (Carlslab, CA, USA); sodium dodecyl sulfate was obtained from J.T. Baker (Mallinckroft Baker, Inc., Phillipsburg, NJ, USA); bromophenol blue, dimethyl urea, hydrogen peroxide, 2-mercaptoethanol, methyl thiazolyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), nonidet p40, phenylmethanesulfonylfluoride (PMSF) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibodies recognizing phosphorylated Thr-202/Tyr-204-extracellular signal-regulated kinase (ERK1/2); ERK1/2 (p-ERK1/2), p38, Tyr-182-phosphorylated p38 MAPK (p-p38 MAPK), c-PKCα, p-cPKCα (Ser 657), phosphotyrosine and luminol reagent were purchased from Santa Cruz (Santa Cruz, CA, USA). Bisindolylmaleimide II, calphostin C, chelerythrine chloride, staurosporine from *Streptomyces* sp., [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole] (SB203580), and [2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyrano-4-one] (PD98059) were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture

HGF cells were obtained from a sample of healthy tissue from patients presenting at the Exodontia Clinic after providing informed consent (Format FBQ-LIFO-001 ISO 9001:2000). The protocol for our study in humans was approved by the Ethical Committee of the Universidad Nacional Autónoma de México. The human gingival fibroblasts, were obtained after informed consent from healthy young individuals (18–20 years) in need of premolar extraction. Gingival tissue was isolated at the cemento-enamel junction of the extracted tooth by means of a surgical blade. The harvested tissue was rinsed several times in Dulbecco's modified Eagle medium containing antibiotics (penicillin 100 U/ml; streptomycin 125 μg/ml and amphotericin 5 μg/ml). The tissue was cu tinto small pieces and cultured with a medium containing 10% foetal bovine serum. When the cells that grow from the explants had reached confluence, they were detached with 0.025% (w/v) trypsin in PBS for 10 min and subcultured in flasks. Cells that remained attached to the bottom of the flash were discarded in order to avoid contamination by epithelial cells, which are not as easily detached as fibroblasts. HGF were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell cultures used in all experiments were between passage 5 and 15 (Bartold, 1987).

2.3. Cell viability assay

Cell viability was determined using a MTT assay (Denziot and Lang, 1986). Cells were gently washed with DMEM and exposed to H_2O_2. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. Cells were incubated for 2 h at 37 °C, media was removed and the formed formazan crystals in viable cells were solubilized with 100 μl of DMSO. Assays were performed in a 96-well plate and the absorbance of each well was measured at 550 nm with ELISA reader (BioTek ELx808). Data were expressed as a percentage of control measured in the absence of H_2O_2.

2.4. Immunocytochemistry

Cells were grown on glass coverslips and fixed for 30 min with 2% formaldehyde in PBS at 4 °C. Cells were then incubated with 0.1% Triton X-100 in PBS for 5 min, followed by five washes in PBS. For visualization of ERK1/2, cells were treated with antibody to ERK1/2 (diluted 1:100 in PBS) for 1 h, washed five times with PBS, followed by incubation with rhodamine-conjugated goat anti-mouse IgG (diluted 1:100 in PBS) for 45 min. All the staining was carried out at room temperature. The preparations were mounted in resin and examined with a confocal photomicroscope. The experiments were performed five separate times.

2.5. Trypan blue exclusion method

The effects of hydrogen peroxide on cell growth and death were determined by the trypan blue exclusion method as we described previously. Cells, seeded in 6-well plates, were treated in the absence or presence, hydrogen peroxide for various time points. Then, cells were trypsinized and diluted in phosphate-buffered saline (PBS). The cells were then counted using a hematocytometer in the presence of trypan blue solution at a 1:1 ratio (v/v) (Sigma).

2.6. Western blot analysis

Human gingival fibroblasts (HGF) (1×10^6 cells/well) were grown in 6-well plates. The cells were serum starved for 16 h and treated with hydrogen peroxide in DMEM with 2% foetal bovine serum, after the treatment, the media was aspirated, cells were washed twice with PBS, and 50 μl of cold lysis buffer was added. Lysis buffer: 0.05 m tris–HCl, pH 7.4, 0.15 M NaCl, 1% nonidet P-40, 0.5 M PMSF, 10 μg/ml leupeptin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate, all obtained from Sigma Chemical Co. (St. Louis, MO, USA). The cells were removed from the plate by scraping and the lysate was transferred to a microcentrifuge tube and pulse sonicated (1 s × 30) on ice. Western blot analysis for the presence of phosphorylated proteins was performed on whole cell extracts. The protein concentrations in the cell extracts were measured using Ponceau (Sigma Chemical Co., St. Louis, MO, USA). Protein (50 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.05% bromophenol blue, and 1.25 M tris–HCl, pH 6.8; all obtained from Sigma Chemical Co). Equal amounts of protein resolved by 10% SDS–PAGE. Protein was transferred to nitrocellulose (Bio-Rad, Hercules, California, USA) overnight at 30 V. The membrane was blocked with 150 mM NaCl, 100 mM tris–HCl and 5% bovine serum albumin for 1 h, washed with TBST and then incubated with anti-phosphotyrosine mouse monoclonal IgG (1:1000), anti-p-ERK (Tyr-204 phosphorylated) mouse monoclonal IgG (1:1000), or phospho-PKCα rabbit polyclonal IgG (Ser 657) (1:1000) (upstate) overnight at 4 °C. Blots were washed three times with TBST and incubated for 1 h with HRP-conjugated anti-mouse IgG Ab (1:1000) or HPR-conjugated anti-rabbit IgG Ab (1:1000). Immunoreactive bands were developed using a chemiluminescent reaction and bands visualized by autoradiography. The localized formation of peroxide through reaction of hydrogen peroxide with immobilized horseradish peroxidase is widely used in the western blotting system.